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Calcium channel blocker as a drug candidate for the treatment of generalised epilepsies

Final degree project

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ABBREVIATIONS

AED	antiepileptic drug
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANNA-1	antineuronal nuclear antibody 1
BBB	blood-brain barrier
Bn	benzyl
BnBr	benzyl bromide
BnNCO	benzyl isocyanate
Boc	*tert*-butoxycarbonyl
Bu_4NBr	tetrabutylammonium bromide
Ca^{+2}	calcium ion
CACNA1	calcium channel voltage-dependent gene
cAMP	cyclic adenosine monophosphate
CCB	calcium channel blocker
cGMP	cyclic guanosine monophosphate
CH_3CN	acetonitrile
Cl^-	chlorine ion
C_{max}	maximum concentration
CMV	cytomegalovirus
CTScan	computed axial tomography
DCM	dichloromethane
DIPEA	*N,N*-diisopropylethylamine
DMF	dimethylformamide
DMPK	drug metabolism and pharmacokinetics
DNET	dysembryoplastic neuroepithelial tumours
EEG	electroencephalogram
EPSP	excitatory post-synaptic potential
FDA	food and drug administration
Fe	iron
FLIPR	fluorescence imaging plate reader
fMRI	functional magnetic resonance imaging
GABA	γ-amino-α-hydroxybutyric acid
GAD65	glutamic acid decarboxylase 65
GAERS	generalised absence epilepsy rat of Strasbourg
GluR5	kainate receptor
GTC	generalised tonic-clonic
H^+	hydrogen ion
H_2	hydrogen
H_2O	dihydrogen dioxide (water)
HATU	1-[bis-(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxide hexafluorophosphate
HCl	hydrochloric acid
HCOOH	formic acid
HIV	human immunodeficiency virus
HLM	human liver microsomes
HPLC	high-performance liquid chromatography
HSE	herpes simplex encephalitis
HTS	high-throughput screening

HVA	high voltage-activated
IC_{50}	half maximal inhibitory concentration
IGE	idiopathic generalised epilepsies
ILAE	international league against epilepsy
K^+	potassium ion
K_2CO_3	potassium carbonate
KCNQ2-5	potassium voltage-gated channel subfamily Q member 2 type 1
LTS	low threshold spikes
MEG	magnetoencephalography
MeLi	methyl lithium
MeOH	methanol
$MgSO_4$	magnesium sulfate
MRI	magnetic resonance Imaging
MW	molecular weight
Na^+	sodium ion
NaH	sodium hydride
Net_3	triethylamine
NH_4Cl	ammonium chloride
NMDA	*N*-methyl-*D*-aspartate
NMDAR	*N*-methyl-*D*-aspartate receptor
NMP	*N*-methyl-2-pyrrolidone
nRT	neuronal response telemetry
Pd/C	palladium on carbon
$Pd_2(dba)_3$	tris(dibenzylideneacetone)-dipalladium(0)
PET	positron emission tomography
PK	pharmacokinetics
po	per os (oral administration)
RLM	rat liver microsomes
rt	room temperature
SAR	structure and activity
SE	status epilepticus
S_NAr	nucleophilic aromatic substitution
SPECT	single-photon emission computed tomography
SV2A	synaptic vesicle protein 2A
THF	tetrahydrofuran
TTCC	T-type calcium channel
TTCCB	T-type calcium channel blocker
VGCC	voltage-gated calcium channels
VGKC	voltage-gated potassium channel
X-PHOS	2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl
WAG/Rij	Wistar Albino Glaxo from Rijswijk-rat

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1. ABSTRACT

Epilepsy represents one of the most common chronic brain diseases affecting more than 50 million people worldwide. The current available antiepileptic drugs (AEDs) present multiple mechanisms of action whether they are direct or indirect, exerting their function in modulating several ion channels. The most common channels implicated are $GABA_A$-receptors, voltage-gated sodium channels (VGSC), voltage-gated potassium channels (VGKC), high-voltage-gated calcium channels (HVGCC) and AMPA receptor. *Actelion Pharmaceuticals* targeted another type of ion channel whose threshold was low, differing from the classical HVGCC: T-type calcium channels (TTCCs). These low-threshold activation channels are products of different genes in mammals, being *CACNA1H* the most relevant in epileptogenesis. The unclassified epilepsies are categorised by 'idiopathic', and there is a strong bond between genetic variation in the T-type calcium channel gene *CACNA1H* and idiopathic generalised epilepsies (IGEs). The different TTCCs are Ca_v3.1, Ca_v3.2 (*CACNA1H*) and Ca_v3.3, whose role in neuronal excitability and muscle contraction is crucial. However, these calcium antagonists have traditionally been used for the treatment of cardiovascular diseases, and further optimisation of their poor specificity led to a promising biological activity toward the Ca_v3 channel for epilepsy treatment. Currently, there are approximately 28 AEDs commercially available to patients, however, due to the diverse physiological and pathological processes implicated in this brain disorder, more than 30% of epileptic patients are pharmacoresistant to therapy and thus, unable to achieve seizure control. Despite the plethora of the approved AEDs, there is likely a limited number of actual functional targets relevant to epilepsy, and it calls for novel compounds with new mechanisms of action.

In this work is detailed the synthesis of a 1,4-benzodiazepine as a new brain penetrant, selective, triple TTCC blocker. This new molecule is **(3*R*,5*S*)-11** [(3*R*,5*S*)-*N*,1-dibenzyl-3,5-dimethyl-1,2,3,5-tetrahydro-4*H*-pyrido-[3,4-e][1,4]diazepine-4-carboxamide]. Furthermore, *Idorsia Pharmaceuticals* characterized another promising molecule that reached clinical trials: *N*-{1-[(5-cyanopyridin-2-yl)methyl]1*H*-pyrazol-3-yl}-2-{4-[1,1,1-(trifluoromethyl)cyclopropyl]-phenyl}acetamide (**12**). Compound **12** (ACT-709478) is synthesised in this project by making react aminopyrazole **28** with 2-{4-[1,1,1-(trifluoromethyl)cyclopropyl]phenyl}acetic acid (**24**). Both compounds (**(3*R*,5*S*)-11**, **12**) showed excellent efficacy on the WAG/Rij rat (Wistar Albino Glaxo from Rijswijk-rat) and GAERS (generalised absence epilepsy rat of Strasbourg) models, both genetic models of generalized epilepsy in rodent animals, demonstrating a strong decrease in the epileptic activity due to an excellent TTCC blockade.

RESUM

L'epilèpsia representa una de les malalties cròniques del cervell més comunes afectant més de 50 milions de persones a tot el món. Els actuals fàrmacs antiepilèptics presenten múltiples mecanismes d'acció, ja siguin directes o indirectes, exercint la seva funció en la modulació de diversos canals iònics. Els canals més freqüents implicats són els receptors $GABA_A$, els canals de sodi dependents de voltatge (VGSC), els canals de potassi voltatge dependents (VGKC), els canals de calci d'alt voltatge dependents (HVGCC) i el receptor AMPA. L'empresa farmacèutica *Actelion* es va proposar com a objectiu uns altres tipus de canals iònics: els canals de calci de tipus T (TTCCs), el llindar dels quals era baix, diferint així dels clàssics canals de calci d'alt voltatge. Aquests canals de baix llindar d'activació són producte de diferents gens presents als mamífers, essent així CACNA1H el més rellevant quant a epileptogènesi. Les epilèpsies no classificades són categoritzades com a "idiopàtiques" i existeix un fort enllaç entre la variació genètica del gen CACNA1H en els canals de calci tipus T i les epilèpsies generalitzades idiopàtiques (IGEs). Els diferents tipus de TTCCs són $Ca_v3.1$, $Ca_v3.2$ (*CACNA1H*) i $Ca_v3.3$, el rol dels quals és crucial en l'excitabilitat neuronal i la contracció muscular. No obstant això, aquests antagonistes del calci han estat utilitzats tradicionalment per al tractament de malalties cardiovasculars i una optimització addicional de la seva pobra especificitat va portar una activitat biològica prometedora envers el canal Ca_v3 pel tractament de l'epilèpsia. Actualment, hi ha aproximadament uns 28 AEDs disponibles comercialment per als pacients, malgrat això a causa dels processos fisiològics i fisiopatològics implicats en el procés de l'epilèpsia més del 30% d'aquests pacients epilèptics són fàrmacoresistents a la teràpia i, per aquest motiu, incapaços d'aconseguir un control de les convulsions. Tot i haver una sobreabundància de d'AEDs aprovats, hi ha una limitació de dianes funcionals rellevants per a l'epilèpsia i això requereix compostos novells nous amb mecanismes d'acció nous.

En aquest treball la síntesi de 1,4-benzodiazepina està detallada com un nou compost, amb capacitat penetrant del cervell, selectiu i capaç de blocar els tres tipus de TTCCs. Es tracta de la molècula **(3*R*,5*S*)-10** [(3*R*,5*S*)-*N*,1-dibenzil-3,5-dimetil-1,2,3,5-tetrahidro-4*H*-pirido-[3,4-*e*][1,4]diazepina-4-carboxamida]. Addicionalment, l'empresa farmacèutica *Idorsia* va caracteritzar una altre molècula prometedora que va aconseguir arribar a assajos clínics: *N*-{1-[(5-cianopiridina-2-il)metil]1*H*-pirazol-3-yl}-2-{4-[1,1,1-(trifluorometil)ciclopropil]-fenil}acetamida (**11**). El compost **11** (ACT-709478) és sintetitzat en aquest projecte fent reaccionar l'aminopirazol **25** amb l'àcid 2-{4-[1,1,1-(trifluorometill)ciclopropil]fenil} acètic (**23**). Els dos compostos (**(3*R*, 5*S*)-10**, **11**) van mostrar una eficàcia excel·lent en els models de ratolí WAG/Rij (Wistar Albino Glaxo de Rijswijk-rat) i GAERS (rata d'epilèpsia generalitzada d'Estrasburg), ambdós models d'epilèpsia generalitzada en animals rosegadors, demostrant així una forta disminució de l'activitat epilèptica a causa d'un excel·lent blocatge dels canals TTCC.

2. AREAS INCLUDED

This undergraduate thesis encompasses the main subject, which is Organic Chemistry. At the end of this project, it is schematically shown one of the possible pathways to synthesise new compounds as potent T-type calcium channel blockers by organic reactions. This subject is concerned with carbon-like compounds in living organisms and nowadays extended to human-made substances, chemical reactions and molecule properties.

Another area included in this project is Molecular Biology, a field of science that has to do with the molecular basis, chemical structures and processes of biological phenomena as well as the regulation of different interactions. Since in this work it is explicitly explained how ion channels related to calcium work, the integration of this area becomes crucial to understand the mechanism of action of different antiepileptic drugs and newer molecules.

This final degree project also covers Physiology and Pathophysiology due to the relevance of how epilepsy works and which processes of the organism change from normal conditions. Not only is essential for this reason but also for a good comprehension of how antiepileptic molecules exert their effect on different targets. While physiology is a medical discipline that describes normal functions, parts and processes of living organisms, pathophysiology is the study of a deranged role in an individual or an organ associated with a disease or syndrome.

Last but not least, Pharmacology and Therapeutics is equally relevant to Organic Chemistry since both subjects represent the main fields of this project. Pharmacology is essential for the study of drug actions, drug discovery, their use and method of administration in the treatment of a disease. Therapeutics is more considered to be the science of healing that deals with the treatment of a disease. Taking into account that two new drug candidates for the treatment of epilepsy are going to be explained throughout this work, this last subject is notoriously indispensable to complete the project.

3. INTRODUCTION

Epilepsy can start at any age and affects millions of people worldwide. It is a complex brain disorder characterised by an abiding predisposition to generate epileptic seizures, which may present diverse phenotypes comprehending from a brief loss of consciousness or muscle spasms up to severe and extended convulsions._The term 'epilepsy' should be considered as a set of diseases included in the nervous system that encompasses many seizure disorders and consequent syndromes that may be caused by various aetiologies. Clinical presentations and aetiologies are so diversified that the existing epileptic syndromes are classified in nearly 60 different classes.

The molecular basis of epilepsy is complex due to the numerous types of targets affected when epileptogenesis appears. There is an increase of the neuronal excitability and a decrease of the inhibitory activity because the glutamate is more elevated, and GABA remains reduced. Since there is an increasing pharmacoresistance to the therapy even trying different

AEDs in combination, a new strategy has been assessed in clinical trials that consist of optimising calcium antagonists commonly used for hypertension and heart angina. On a first attempt, these drugs were added to the standard antiepileptic therapy using AEDs. However, it was later demonstrated that improving the properties of these cardiovascular molecules, drug metabolism and pharmacokinetics could show an excellent effect in epilepsy treatment by blocking T-type calcium channels.

In this work, it is explained the link between these channels and how its inhibition enables a seizure suppression. After bibliographic research, I propose the synthesis of two promising antiepileptic molecules that have entered clinical trials. Researchers started from compounds with poor physicochemical and pharmacological properties, and by improving their solubility, lipophilicity and metabolic stability led to final molecules whose synthesis are suggested throughout this project.

4. OBJECTIVES

The principal objective of this bibliographic project is to know the relation between calcium ion channels and their effect on epilepsy and to settle a synthetic pathway of two selected new compounds with promising effects on seizure suppression in epilepsy. Since the majority of calcium antagonists have been used for the treatment of cardiovascular diseases, in this work the aim is to know how a proper synthesis and molecule optimisation of a calcium channel blocker, is able to show high efficacy and optimum properties in generalised epilepsies.

These hits selected for further optimisation, have been structurally similar to antihypertensive drugs, whose properties improvement led to new promising, brain penetrant, triple T-type calcium channel blockers and thus, became new candidates with new indications for epilepsy treatment.

5. METHODS

In this report, I propose a synthetic pathway to achieve new compounds with promising effects on the treatment of epilepsy. The synthesis has been possible due to the bibliographic research in scientific databases I have used. On the one hand, I have researched on the free platform PubMed®. Moreover another useful resource for my project it has been MEDLINE®, the National Library of Medicine® (NLM), the American Chemical Society® (ACS), the British Journal of Clinical Pharmacology® (BJCP) and the Epilepsy Foundation website. These platforms have provided the majority of bibliographic information; however, without a chemistry database like SciFinder®, it could not have been possible to develop this project since Organic Chemistry is the main covered area. This portal contains a huge database and literature from many scientific disciplines, which allows users to explore all properties and related information with chemical substances.

Lastly, by using the ChemDraw® programme, I have been able to draw the chemical structure of all the explicit molecules in the project as well as their respective reactions depending on the section.

6. GENERAL FEATURES OF EPILEPSY

6.1. Definition

Epilepsy is defined as a neurological condition characterised by an enduring predisposition to generate recurrent seizures which may be unprovoked by any identifiable cause. An epileptic seizure is a brief episode of signs and/or symptoms caused by an unusual and excessive discharge of a set of neurons on the brain, and it ceases spontaneously[1]. Although the term 'epilepsy' is currently known as a single disease entity, it should be more considered as a symptom of a neurological disorder in the same manner for a seizure as a clinical manifestation[2].

Correctly defining the term of epilepsy, it is described as a set of diseases included in the nervous system caused by the alteration of the brain's electrical activity provoking immediate symptoms and usually accompanied by losing consciousness. Excessive neuronal hyperactivity causes adjacent transitory modifications of behaviour, perception or mobility. Symptoms may vary depending on the beam of affected neurons during the seizure[2].

Multiple, interacting factors contribute to the totality of epilepsy for an individual patient, for instance, a specific aetiology, genetic background, associated neurologic abnormalities, personality development and psychosocial adjustment, site of brain dysfunction, cognitive abilities, seizure activity, age of onset and duration as well as environmental factors[3].

6.2. Classification of epilepsies

The International League Against Epilepsy (ILAE) is a worldwide organisation founded in 1909 whose goals are the advancement and dissemination of knowledge about epilepsy, the improvement services, care for patients and the promotion of research, education and training. This grouping managed to update classifications of seizures and epilepsies since previous terms were not as technical and precise as current ones. It limited clinicians and researchers to find an accurate care term to enhance communication and discussion and enable patients to understand their clinical condition[4].

Table 1. General classification and aetiology of epilepsies

<table>
<tr><th>EPILEPSY TYPE</th><th>AETIOLOGY</th><td rowspan="5">EPILEPSY SYNDROMES</td></tr>
<tr><td>Focal</td><td rowspan="4">Structural
Genetic
Infectious
Metabolic
Immune
Unknown</td></tr>
<tr><td>Generalised</td></tr>
<tr><td>Combined generalised and focal</td></tr>
<tr><td>Idiopathic (unknown)</td></tr>
</table>

Since epilepsy is a disease that comprehends a collective of syndromes, there is a broader classification of epilepsies regarding its diverse clinical manifestation and the specific syndromes included in it (*see Table 2*).

Table 2. Detailed classification of epilepsies: epilepsy type, aetiology and syndromes[3]

Localization-related (focal)	Idiopathic	Benign childhood epilepsy with centrotemporal spikes Childhood epilepsy with occipital paroxysms Primary reading epilepsy
	Symptomatic	Chronic progressive epilepsia partialis continua of childhood
	Cryptogenic[1]	The symptomatic and cryptogenic categories constitute syndromes of wide individual variability that are based principally on: A. Seizure types B. Anatomic localisation ▪ Temporal lobe epilepsies ▪ Occipital lobe epilepsies ▪ Frontal lobe epilepsies ▪ Parietal lobe epilepsies
Generalised	Idiopathic	▪ Benign myoclonic epilepsy in infancy ▪ Childhood absence epilepsy (pyknolepsy) ▪ Juvenile absence epilepsy ▪ Juvenile myoclonic epilepsy ▪ Epilepsy with grand mal (GTC) seizures on awaking ▪ Other idiopathic generalised epilepsies not defined above ▪ Epilepsies with seizures precipitated by specific modes of activation
	Cryptogenic or symptomatic (in order of age)	West-syndrome (infantile spasms) Lennox-Gastaut syndrome Epilepsy with myoclonic-astatic seizures Epilepsy with myoclonic absences
	Symptomatic	Nonspecific aetiology: ▪ Early myoclonic encephalopathy ▪ Early infantile epileptic encephalopathy with suppression-burst ▪ Other symptomatic generalised epilepsies not defined above
		Specific syndromes

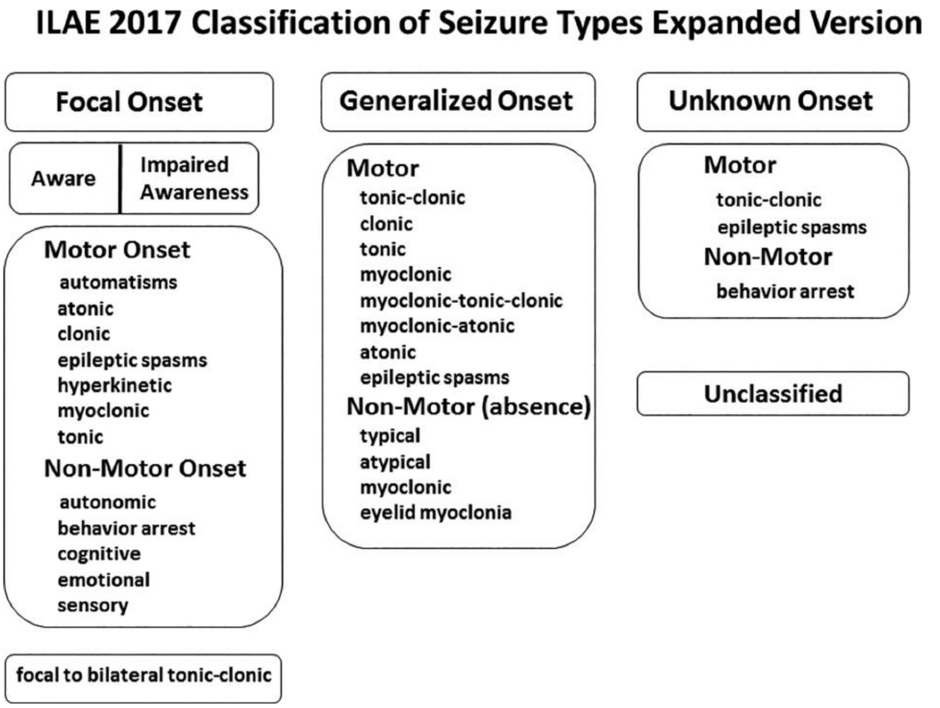
[1]Presumed to be symptomatic but with unknown aetiology.

Epilepsies and syndromes undetermined as to whether focal or generalised	With both generalised and focal seizures	▪ Neonatal seizures ▪ Severe myoclonic epilepsy in infancy ▪ Epilepsy with continuous spike-waves during sleep ▪ Acquired epileptic aphasia (Landau-Kleffner syndrome) ▪ Other undetermined epilepsies not defined above
	Without unequivocal generalised or focal seizures	
Special syndromes	Situation-related seizures	▪ Febrile convulsions ▪ Isolated seizures or isolated status epilepticus (SE) ▪ Seizures due to acute metabolic or toxic factors such as alcohol, drugs, eclampsia, etc.

6.3. Classification of seizures

Since seizures are one of the main clinical disorders of epilepsy and comprehend a vast manifestation depending on the person experiencing it, the ILAE managed to classify different types of seizures depending on the location of the brain disorder, muscular response and its origin.

Figure 1 ILAE 2017 classification of different seizure types[4,5]



6.4. Aetiology, physiology and pathology in epilepsy

6.4.1. Aetiology

The aetiology of this neurological disorder resides in multifactorial causes such as structural brain alterations with traumatic or non-traumatic antecedents for instance: ictus, cranial trauma or brain tumour. Moreover, genomic mutations, immunological disorders and metabolic changes, like porphyria, may cause epilepsy as well. The most common cause of non-genetic epilepsy is an infectious disease, which affects the brain; for example, brain tuberculosis or cysticercosis. Not only known disorders are the main responsible for causing the disease but also idiopathic causes are still highly prevalent in current diagnosis despite the diagnostic tests breakthroughs, leading to an undefined and unknown cause of the disease that difficult clinicians to establish a proper diagnostic[6].

The ILAE has defined six etiologic categories which are not hierarchically ordered and more than one might often apply[4].

1. Structural aetiology

Seen by neuroimaging, an abnormal screening of an electroencephalogram (EEG) with discordant seizure semiology may suggest its starting point. Nevertheless, it is likely unrelated to the patient's epilepsy and would not be considered relevant when classifying their epilepsy type[3].

2. Genetic aetiology

Having a relevant family history and typical characters (seizure semiology, EEG) without the presence of molecular genetics is sufficient for a genetic aetiology classification. Thus, a specific variant in a gene or copy number variant, known to be pathogenic for epilepsy, would lead to a genetic cause. However, genetic disease-variants often arise *de novo* because of mutations and are not inherited, so despite a family history of epilepsy not always will a patient have a genetic cause for its epilepsy[3].

3. Infectious aetiology

The usual cases of infectious epilepsy are caused by neurocysticercosis, human immunodeficiency virus (HIV), cytomegalovirus (CMV) and cerebral toxoplasmosis. Patients with seizures due to a resolved infection (e.g. meningitis) are classified as having an infectious cause of their epilepsy. Regarding non-resolved infections may be considered a structural aetiology as an acute infection[3] provokes its seizures.

4. Metabolic epilepsies

A metabolic disorder can lead to an acute-symptomatic seizure (e.g. cerebral folate deficiency and pyridoxine-dependent seizures). Many metabolic epilepsies are genetic in terms of aetiology, as metabolic derangements mainly are inherited[3].

5. Immunaetiology

Autoimmune disease is the leading cause of new-onset epilepsy (e.g. autoimmune encephalitis). There is proof that autoimmune encephalitis and epilepsy have been linked to both neuronal intracellular (GAD65, ANNA-1, and Ma) and neuronal cell surface antibodies (VGKC, NMDAR, AMPA, GABA-B, complex and GluR5)[7,8].

6. Unknown category (idiopathic)

Aetiology for these patients remains undefined and unclear.

6.4.2. Pathophysiology

Epileptic syndromes differ pathophysiologically despite sharing common ictogenesis features such as increased neuronal excitability and synchronicity, as well as mechanisms involved in interictal-ictal transition[9]. Alterations of synaptic functions and inherent properties of neurons are common mechanisms of hyperexcitability. Furthermore, due to molecular genetics research and progress, it has been pointed and hypothesis about mutations of genes encoding ion channels in some forms of human epilepsy[9].

Epileptic seizures arise from a sustained abnormal discharge of a group of neurons associated with a variety of causative factors such as trauma, neoplasms (e.g. Pleomorphic Xanthoastrocytoma, ganglioglioma, DNETs which are explained next below), infections, metabolic derangements, oxygen deprivation and inflammatory disorders of the cerebral hemispheres[3]. A neoplasm called Pleomorphic Xanthoastrocytoma is a superficially growing lesion with a meningeal component in young and middle-aged people[10]. Gangliogliomas are an admixture of neoplastic glial elements and disorganised cells including binucleate or multinucleate forms[11]. Regarding the acronym DNET, it stands for dysembrioplastic neuroepithelial tumour, a slow-growing glioneuronal neoplasm with a cortical or deep grey matter location that shows a powerful association with partial seizures[12]. An infection can cause a chronic inflammatory disorder, for instance, in the acute or chronic stages of herpes simplex encephalitis (HSE)[8]. However, the most common rare type of encephalitis designed to cause seizures is Rasmussen's encephalitis, which appears as a juvenile form of epilepsy associated to a progressive neurologic deficit characterised by mental retardation and cognitive dysfunction[8]. Neuropathology of epilepsy depends on disorders of neuronal migration, genetic predisposition, spontaneous gene mutations and common structural defects of the brain[9]. Ictogenesis is the generation of a seizure and arises from multiple non-specific causative factors. Reaffirming that distinct types of epilepsy differ in pathophysiology, numerous causes may originate neuronal hyperexcitability in ictogenesis[9].

Firstly, excitability arising from individual neurons happens in the post-synaptic membrane altering the character of receptor protein and thereby developing paroxysmal depolarisation. An increase of Ca^{+2} conductance takes place when epileptic neurons are activated[9]. Thus, the efficacy and the number of calcium channels is permanently increased. Secondly, not only individual neurons but also excitability from a neuronal microenvironment may cause ictogenesis as well. Functional and structural alterations occur at the same time in an epileptic seizure. Changes in neurotransmitter levels, concentrations of cations and anions and metabolic mutations involve both neurons and glia in structural changes[9]. For instance, excessive extracellular K^+ depolarises neurons and leads to spike discharge. Thereby, changes in extracellular Ca^{+2} (decrease) during a seizure, precede those of K^+ (increase), and calcium levels return to normal more quickly than K^+ [3].

Different mechanisms are involved in interictal-ictal transition whether it is a nonsynaptic or synaptic mechanism[3].

Nonsynaptic mechanisms[3]

1. Alterations in ionic microenvironment: some neurons are very sensitive to changes in membrane currents (e.g. increased extracellular K^+, decreased extracellular Ca^{+2})
2. Decreases in the size of extracellular space
3. Failure of ion transport: Na^+- K^+ pump or Cl^- - K^+ co-transport
4. Presynaptic terminal bursting
5. Ephaptic[2] interactions

Synaptic mechanisms[3]

1. Depression of GABAergic inhibition
2. NMDA receptor activation, voltage-dependent EPSPs (excitatory postsynaptic potentials)
3. Frequency potentiation of EPSPs
4. Actions of modulations

In terms of biochemistry, a seizure is provoked by an alteration of neurotransmitters due to the exaggerated hyperexcitability of neurons. Thereby, there is an inhibition of GABA A receptors and at the same time activation of glutamate receptors[3,9]. Metabolic pathways connect these two metabolites. In addition, the neuron sends the signal through voltage-gated Na^+/K^+ ion channels that will arrive at the synaptic terminal where Na^+ and Ca^{+2} channels will be allocated leading to a positive potential membrane. Consequently, calcium will diffuse through ion channels and will provoke the release of neurotransmitters outside the terminal[1,4].

In order to stop seizures, a blockade of Na^+ and/or Ca^{+2} ion channels and equilibration between both activator (enhance GABA activity) and inhibitory neurotransmitters (decrease glutamate activity) is a general strategy for epilepsy treatment[3,13].

6.5. Diagnostic tests

Expecting a possible diagnosis of epilepsy, a medical evaluation may include a neurological exam and blood tests[14]. The most common tests used to diagnose epilepsy are mentioned below:

- **EEG** (*Electroencephalogram*)
  EEG is a non-invasive and painless diagnostic that measures electrical impulses between brain cells. Electrodes are placed on the scalp, and the frequency of these impulses are measured and recorded on a graph. Any abnormalities on the brain waves can be used to identify the presence, location and severity of the seizures[15,14].

- **MRI** (*Magnetic Resonance Imaging*)
  A non-invasive diagnostic test that uses a magnetic field to measure changes in the brain. This technique provides detailed cross-sectional images that enable to detect any structural abnormalities, and it is considered to be the most important test when diagnosing

[2] Ephaptic interactions occur when currents from activated neurons excite adjacent neurons.

epilepsy because of its accurate brain representation. The procedure becomes invasive when contrast dye may be administrated to provide clearer images[15,14].

- **fMRI** (*Functional MRI*)
  Measures the changes in blood flow that occur when specific regions of the brain are working. It is commonly used before surgery to identify with precision the exact locations of critical functions (e.g. speech and movement) so that surgeons avoid any injuries in those regions while operating[15,14].

- **SPECT** (*Single-Photon Emission Computed Tomography*)
  This technique is based on imaging, whose purpose is to measure blood flow through the brain. A small amount of low-dose radioactive tracer is injected into a vein to create a detailed third-dimensions map of the blood flow activity in the brain during the seizures. SPECT is not usually necessary for diagnosing epilepsy[15,14].

- **PET** (*Positron Emission Tomography*)
  PET is an imaging technique that measures brain activity through its use of sugar and oxygen. PET scans use a small amount of low-dose radioactive tracer which is injected into a vein and release tiny particles called positrons. This test helps to visualise active areas of the brain and detect abnormalities, and the data enable doctors to determine where the seizures occur[15,14].

- **CTScan** (*Computed Axial Tomography*)
  This technique is a non-invasive and painless test that produces cross-sectional images of different body areas using X-rays. CT scans reveal abnormalities in the brain that might be the cause of the seizure such as tumours, bleeding or cysts[15,14].

- **MEG** (*Magnetoencephalography*)
  A magnetoencephalography is a new test used to generate a representation of the brain's magnetic fields produced by brain activity. The aim is to identify potential areas of seizure onset[15,14].

7. ANTIEPILEPTIC DRUGS (AEDs)

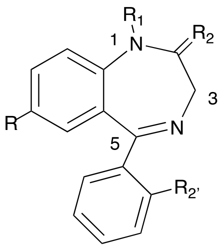
Around approximately 28 antiepileptic drugs are available to patients and have multiple targets. Despite the plenty of AEDs available and their use even in combination therapy, 20-30% of epileptic patients are pharmacoresistant with seizures not appropriately controlled[16]. As knowledge on the pathophysiology of epilepsy, most mechanisms of action of AEDs are based on only a few and basic neurochemical mechanisms. Current AEDs decrease neuronal membrane excitability by interacting with ion channels or neurotransmitter receptor complexes[17]. Drugs that decrease membrane excitability through ion channels interactions act on sodium and calcium channels. Regarding AEDs that interact with neurotransmitter complexes, these can decrease the GABAergic activity by inhibiting its neurotransmission as well as working on the excitatory neurotransmission[3].

7.1. Current approved AEDs and mechanism of action

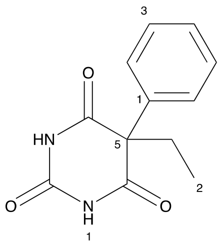
The current antiepileptic drugs available in the market are listed below including its chemical structure and classified by their primary mechanisms of action[17].

Enhance GABA action

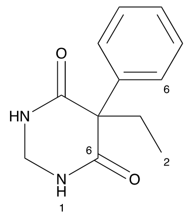
1. BENZODIAZEPINES (long-lasting effect)
- Increase GABA action
- Reduce sustained repetitive discharges



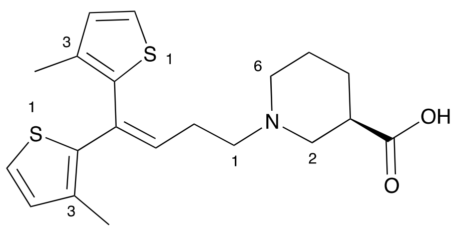
2. PHENOBARBITAL
- Increases GABA action
- Reduces sustained repetitive discharges
- Reduces voltage-dependent Ca^{+2} currents



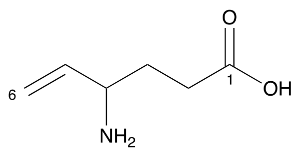
3. PRIMIDONE (structural analogue of phenobarbital)
- Reduces sustained repetitive discharges
- Blocks voltage-dependent Na^{+} currents



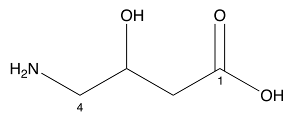
4. TIAGABINE
- Neuronal and glial GABA-uptake inhibitor



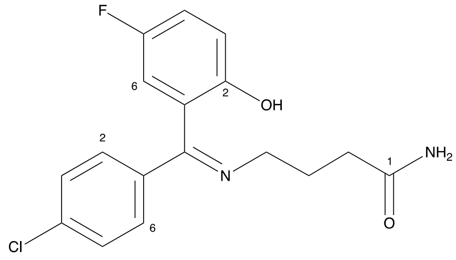
5. VIGABATRIN
- GABA-Transaminase inhibitor
- Inhibits GABA uptake



6. GABOB (γ-amino-β-hydroxybutyric acid)
- Analogue of the neurotransmitter GABA, may function as a neurotransmitter itself

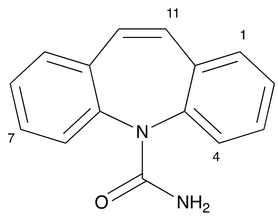


7. PROGABIDE
- GABA agonist at A and B sites

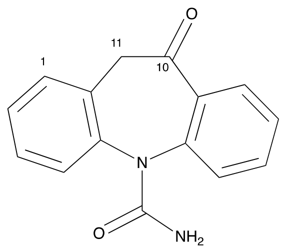


Sodium channel blockers

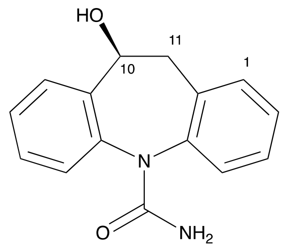
8. CARBAMAZEPINE
- Blocks voltage-dependent Na^{+} channels
- Limitation of sustained repetitive discharges



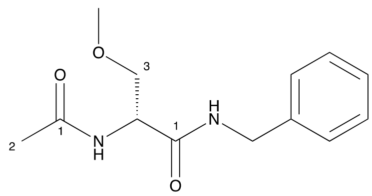
9. OXCARBAMAZEPINE
- Inhibition of voltage-dependent Na^{+} channels
- Inhibition of voltage-activated Ca^{+2} currents



10. ESLICARBAZEPINE
- Inhibition of voltage-dependent Na^{+} channels

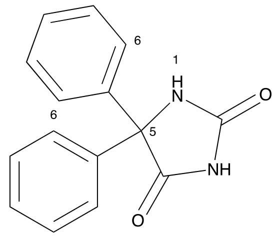


11. LACOSAMIDE
- Inactivates voltage-gated Na^{+2} channels: decreases the sodium levels
- Inhibition of glutamatergic neurotransmission (decreases NMDA action, blocks glycine-site on NMDA receptor)



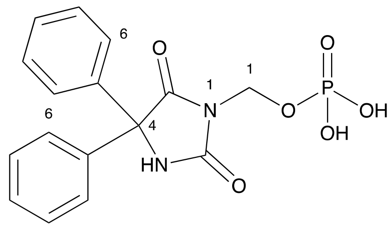
12. PHENYTOIN

- Blocks voltage-gated Na^+ channels
- Reduces Ca^{+2} currents



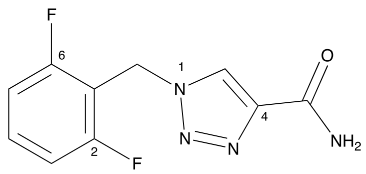
13. FOSPHENYTOIN

- Water-soluble phenytoin used in hospitals



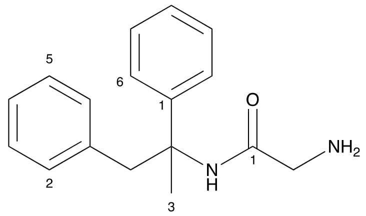
14. RUFINAMIDE

- Prolongation of the inactive state of Na^+ channels



15. REMACEMIDE

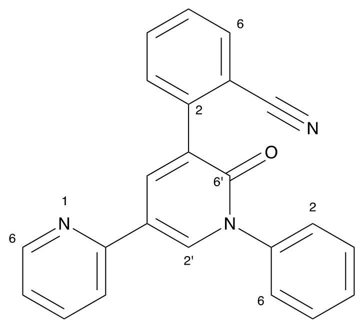
- NMDA receptor antagonist



Glutamatergic inhibitor activity

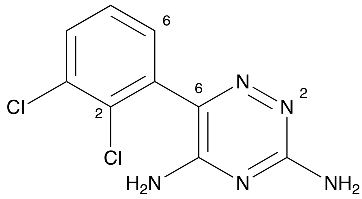
16. PERAMPANEL

- Inhibition of glutamatergic activity. Is a selective non-competitive antagonist of AMPA receptors (ionotropic glutamate receptors)



17. LAMOTRIGINE

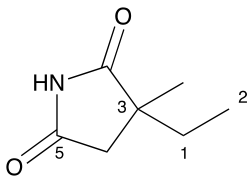
- Reduces glutamate release
- Inhibits voltage-activated Ca^{+2} currents, blocks voltage-dependent Na^{+} channels



Calcium channel blockers

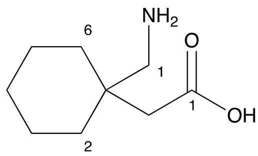
18. ETHOSUXIMIDE

- Reduces T-type Ca^{+2} currents
- Blocks synchronised thalamic discharges



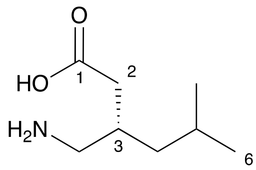
19. GABAPENTIN

- GABA analogue but does not bind to GABA receptors
- Increases synaptic GABA
- May block amino acid transporter
- Binds to voltage-dependent Ca^{+2} channels reducing the intraneuronal concentration of calcium
- Inactivation of Na^{+} channels (N and PQ)



20. PREGABALIN

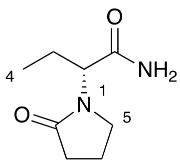
- Binds to voltage-dependent Ca^{+2} channels reducing intraneuronal concentration of calcium



Protein SV2A modulator

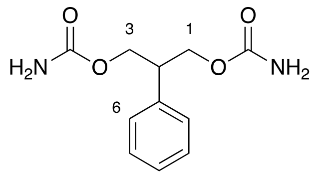
21. LEVETIRACETAM

- Is thought to stimulate synaptic vesicle protein 2A (SV2A), inhibiting neurotransmitter release



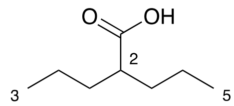
22. FELBAMATE

- Inhibition of glutamatergic neurotransmission (decreases NMDA action, blocks glycine-site on NMDA receptor)
- GABA potentiation
- Blocks voltage-dependent Na^+ channels
- Blocks L-type Ca^{+2} channels



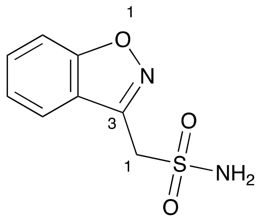
23. VALPROIC ACID (VALPROATE)

- Increases GABA levels by increased synthesis and reduced catabolism
- Blocks T-type Ca^{+2} currents
- Increases Na^+ channel inactivation



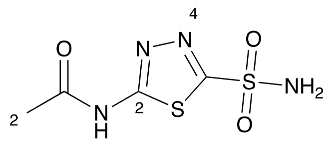
24. ZONISAMIDE

- Blocks Na^+ channels
- Blocks T-type Ca^{+2} channels
- Increases GABA action
- Inhibition of carbonic anhydrase



25. ACETAZOLAMIDE

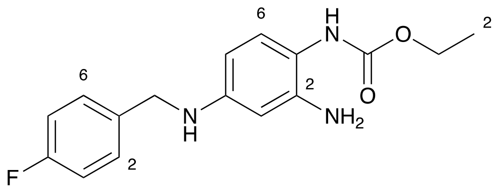
- Inhibits carbonic anhydrase



Others

26. RETIGABINE

- An allosteric modulator of KCNQ2-5 (K_v7.2-7.5) ion channels. First neuronal potassium channel opener for the treatment of epilepsy[18]



Most of AEDs have more than one mechanism of action, and its classification may vary depending on the pharmacological target. For instance, $GABA_A$[19] -receptors are targeted by benzodiazepines, voltage-gated Na^+ channels by carbamazepine, lacosamide, lamotrigine, zonisamide and others; voltage-gated K^+ channels by retigabine[18] and high voltage-gated Ca^{+2} channels by lamotrigine, levetiracetam, topiramate and others[20,3,19].

8. CALCIUM CHANNEL BLOCKERS

8.1. Voltage-gated calcium channels

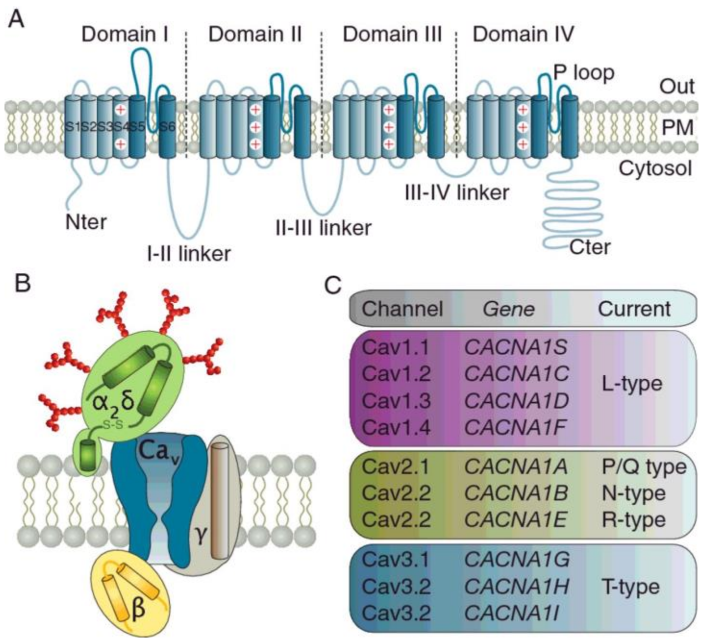
Calcium channels belong to the family of the voltage-gated channels as well as Na^+, K^+ and Cl^- channels, which are permeable to this ions[21]. Aside from this group, it exists another type of channels called ligand-bound ion channels that include these channels but is activated by the extracellular presence of neurotransmitters such as glutamate, intracellular second messengers (e.g. Ca^{+2}) or cyclic nucleotides (e. g. cAMP and cGMP[21]. Most of these channels are allocated in the plasma membrane of all excitable cells, including the neurons of the peripheral and central nervous system[22]. Concretely, calcium channels are highly present in the dorsal root ganglia[23]. These channels control many critical physiological processes, and they allow the influx of calcium into the cytosol which leads to the regulation of several cellular processes like neuronal excitability, pain sensation, muscle contractions, cell development and proliferation and last but not least, hormone and neurotransmitter release[16,23].

Depending on the biophysical and pharmacological characteristics, calcium channels are classified into six different categories according to the required voltage range for their activation. Thus, the types of calcium channels discovered so far are called T, L, N, P, Q or R – type channel[24,3].

Mainly, T-type calcium channels (TTCCs) are functionally distinct from other members of voltage-gated calcium channels (VGCCs) as possess a low voltage-activation[25,23]. In comparison to high voltage-activated (HVA) calcium channels, T-type channels have single-channel conductance, fast activation and inactivation kinetic, hyperpolarised voltage-dependences of activation and inactivation and slow deactivation kinetics[26].

TTCCs are defined by their alpha subunits encoding genes in the mammalian genome (e.g. *CACNA1G* yields to $Ca_v3.1$, *CACNA1H* to $Ca_v3.2$ and *CACNA1I* to $Ca_v3.3$)[22]. T-type calcium channels are mainly found in neurons, glial cells, fibroblasts, osteoblasts, retinal cells, adrenocortical cells as well as having a broad expression pattern including placenta, heart, kidney, smooth muscle, liver and the ovaries[22]. The Ca_v1 family encodes L-type calcium channels and includes different members (from $Ca_v1.1$ through $Ca_v1.4$)[27]. The Ca_v2 family comprehends $Ca_v2.1$, $Ca_v2.2$ and $Ca_v2.3$, which encode P/Q-type, N-type and R-type currents, respectively. On the other side, Ca_v3 includes the family of T-type channels with three subgroups ($Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$)[27].

Figure 2. Molecular composition and diversity of VGCCs[21]

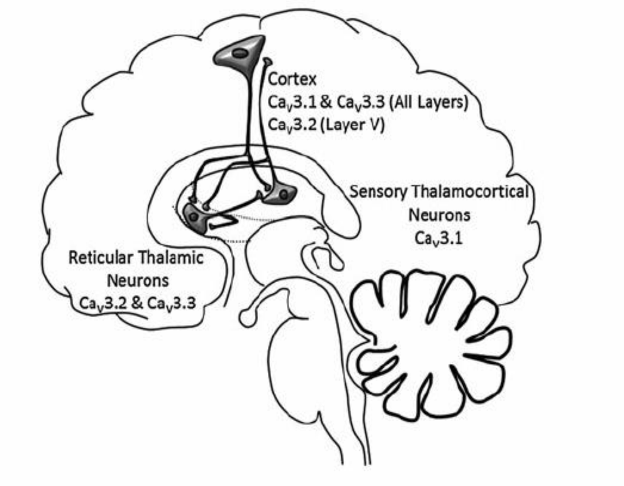


8.2. T-type calcium channels (TTCC) and epilepsy

Calcium T-channels are involved in the regulation of membrane potential and the control of intracellular calcium concentration[24]. In the brain, the three T-type calcium channel subtypes are broadly expressed and play a vital role in the regulation of neuronal excitability[22]. Concretely, TTCCs are located at the cortex ($Ca_v3.1$, $Ca_v3.2$), sensory thalamocortical neurons ($Ca_v3.1$) and reticular thalamic neurons ($Ca_v3.2$, $Ca_v3.3$)[22]. The different types of Ca^{+2} channel α-1 subunits are distributed within neurons, and due to the distinct functional properties, subcellular distributions and subunit composition have different physiological roles and play a major role as contributors to the development of seizures in epilepsy[25]. While P/Q-type channels contribute to generating absence seizures, T-type channels play a crucial role in idiopathic generalised epilepsies and pain[28]. The entry of Ca^{+2} ions through TTCCs leads to depolarization of the membrane and thus, generates low threshold spikes (LTS) in thalamic neurones ($Ca_v3.2$ and $Ca_v3.3$ get involved)[22]. Thalamic neurons belong to a connected circuit that oscillates during natural processes but also can work at improper times as during a generalised seizure. The circuit is constituted by cortical neurons (glutamatergic), thalamocortical neurons (glutamatergic) and thalamic reticular neurons (nRT, GABAergic) and due to the unique voltage dependence of TTCCs allow them to generate low threshold spikes after a post-synaptic potential (inhibitory or excitatory)[29]. These channels remain inactivated at the resting membrane potential of many neurons and recover from an inactivation state during an inhibitory post-synaptic potential[22]. TTCCs open and close at negative membrane potentials and a large amount of Ca^{+2} entries through the channel to intracellular space leading to a high calcium concentration, especially in small compartments such as dendrities[26]. Another property of T-channels is the ability to generate "window currents" when the channel is available to be opened at a given potential instead of being inactivated[22].

Calcium signalling plays a vital role in the survival of neurons, and it is crucial to understand that TTCCs are related to epilepsy because of their high expression in the thalamus[25]. There is a gene that is implicated in the susceptibility to develop epilepsy[30]. Idiopathic generalised epilepsies (IGE) are polygenic disorders caused by mutations of genes. *CACNA1H* and its functional variants are known to be associated with various generalised epilepsy syndromes[31]. Some variants of this gene are responsible for causing generalised absence epilepsies and studies on the Generalized Absence Epilepsy Rat of Strasbourg (GAERS) confirmed these variants are linked to seizure phenotype and can contribute to seizure susceptibility[31]. Pharmacological studies using genetic models of generalised nonconvulsive absence-like epilepsy (the generalised absence epilepsy rat of Strasbourg, GAERS, or the WAG/Rij rat models, Wistar Albino Glaxo from Rijswijk-rat) showed that TTCC blockers had strongly decreased the epileptic activity in these models[24]. Furthermore, mice overexpressing the $Ca_v3.1$ channels present recurrent spike-and-wave discharges whereas knockout mice lacking the $Ca_v3.1$ gene are protected from absence seizures[24].

Figure 3. Distribution of low-voltage-gated TTCCs in the brain[21]



Despite the progress in research about the role of calcium channels in epilepsy, there is not a precise mechanism that confirms how these variants increase seizure susceptibility. Some hypothesis predicted about TTCC variants that might cause changes in gating, and therefore epileptic seizures are: 1) changing the voltage-dependence of activation or inactivation, 2) accelerating channel opening, 3) decelerate channel inactivation, 4) slowing open to close transitions, 5) accelerating recovery and 6) increasing single channel conductance[27]. Many of these properties may be affected by *CACNA1H* variants found in IGE patients. Changes in the channel structure (e.g. variations on the different domains) may also play an important role in seizure susceptibility[27].

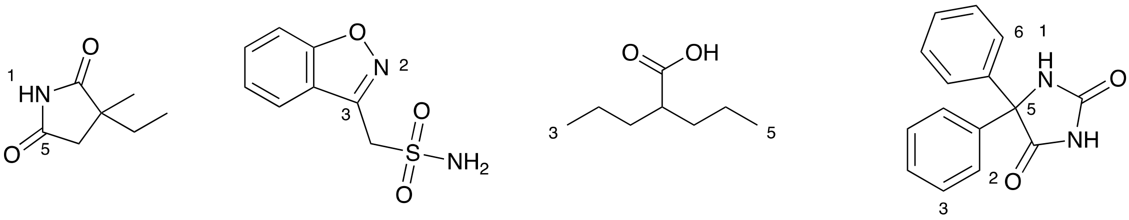
8.3. Molecular structure of calcium channel blockers

T-type calcium channels are known to play roles in the development, preservation and repair of several tissues but also may cause disease when they are not adequately regulated. TTCC blockers have been established to treat several diseases, and the strongest point of these drugs is their safety as well as the efficacy[25]. Aside from their assigned clinical applications, recent studies have found a powerful neuroprotective effect of T-type calcium channel blockers and a useful effect in treating many neurological diseases in animal models[25].

8.3.1. Clinically used AEDs that act on T-type calcium channels

Calcium channels can be modified by many antiepileptic drugs such as ethosuximide, zonisamide, valproate, phenytoin (*see Figure 4*), mibefradil *(see Figure 6)* and others. This last one was withdrawn from the clinical market in 1998 due to drug interactions leading to abnormal heart rhythms[25]. Regarding to its structure, ethosuximide is a succinimide and it has been used for the treatment of absence seizures (generalised nonconvulsive pattern) while zonisamide is chemically classified as a sulphonamide and is used to treat partial seizures, childhood epilepsy and other types of convulsions. These two drugs reach therapeutic concentrations to block the T-type calcium channels and show neuroprotection aswell[25].

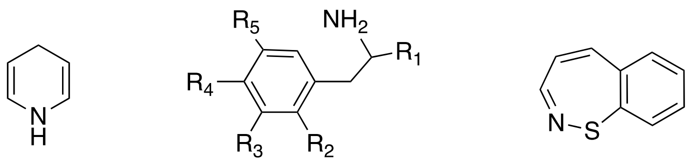
Figure 4. Examples of four antiepileptic TTCC blockers



Ethosuximide Zonisamide Valproic acid Phenytoin

Compared to other AEDs, valproate has a very different structure with an achiral short branched fatty acid with eight carbons that does not include nitrogen or a cyclic ring[25]. Phenytoin (*see Figure 4*) shows the chemical structure of a diphenylimidazolidinedione. In like manner, many antihypertensive/antianginal CCBs where thought to be useful in epilepsy due to its L-type Ca^{+2} channel blockade[25]. Nevertheless, amongst all clinically relevant CCBs to treat cardiovascular disorders, the majority of them are usually able to block both L-type and T-type calcium channel[25]. Dihydropiridines[32], phenylakyilamines[33] and benzothiazepines[25] are the three separate classes of this antihypertensive TTCC blockers drugs.

Figure 5. General structure of antihypertensive TTCC blockers



Dihydropyridine Phenylalkylamine Benzothiazepine

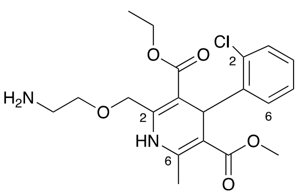
Various antihypertensive/antianginal drugs have clinically assessed as new antiepileptic molecules. Comparing the IC_{50} values between *Table 3* and *Table 4*, there is proof that some of these cardiovascular drugs can block T-type calcium channels in the same threshold than some classical antiepileptic drugs (e.g. ethosuximide and zonisamide in *Table 3*). Zonisamide has a similar IC_{50} to azelnidipine, aranidipine and less resemble amlodipine values. However, bearing in mind that both types of drugs can block TTCC at similar ranges, the next step to optimize these cardiovascular molecules to enter next clinical trials phases is to prove its metabolic stability in liver microsomes, *in vivo* brain penetration, unbound fraction in plasma and brain, animal model testing, pharmacokinetics in the rat and further studies required[24].

Table 3. Data of the TTCC inhibition of two antiepileptic drugs[25]

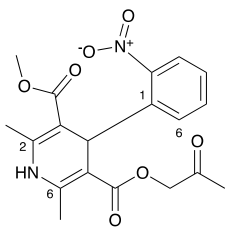
Antiepileptic drug	Ethosuximide	Zonisamide
Pathways inhibited	T-type ($Ca_v3.1$, $Ca_v3.2$); Na	T-type; oxidative; carbonic anhydrase inhibitor
MW (g/mol)	141.17	212.23
IC_{50} (mM) TTCC	0.3-1.0	0.05-0.5

Here below are listed different dihydropyridines being assessed in various countries as new antiepileptic drugs. Some of them have already been approved by the Food and Drug Administration (FDA) in several countries[25].

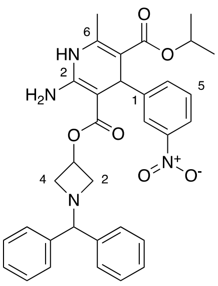
Figure 6. Dihydropyridines and Mibefradil tested as antiepileptic drugs



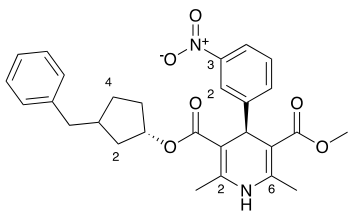
Amlodipine



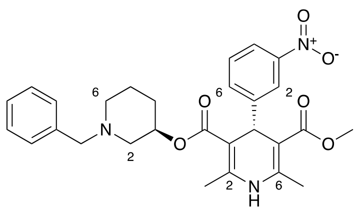
Aranidipine



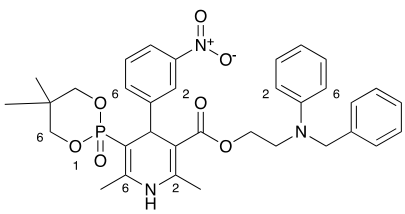
Azelnidipine



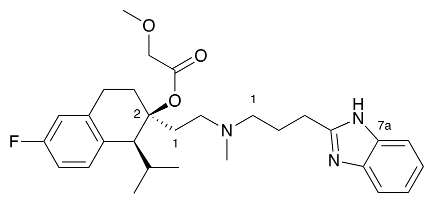
Barnidipine



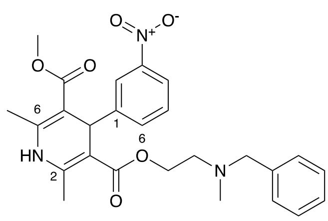
Benidipine



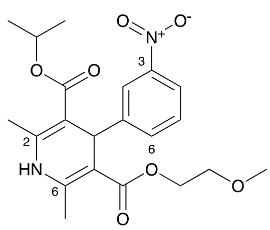
Efonidipine



Mibefradil



Nicardipine



Nimodipine

Table 4. Antihypertensives/antiangina[25]

Drug	**Pathways inhibited**	**MW (g/mol)**	**IC_{50} (mM) TTCC**
Amlodipine	T-type ($Ca_v3.2$ > $Ca_v3.1$ or $Ca_v3.3$), L-type; Na; K	408.88	0.031
Aranidipine[a] CJ	T-type and L-type	388.37	0.03-0.04
Azelnidipine[a] CJ	T-type and L-type	582.65	0.04-0.07
Barnidipine[a] SJO	T-type and L-type	528	0.005-0.02
Benidipine[a] IJCO	T-type and L-type	542.02	0.003-0.2
Efonidipine[a] J	T-type and L-type	631.66	0.0029
Mibefradil[a] O	T-type and L-type; Na; K	568.55	0.00017-0.00029
Nicardipine	T-type and L-type	515.99	0.0028
Nimodipine	T-type and L-type	418.44	0.0056

J **Japan,** ***C*** **China,** ***I*** **India,** ***S*** **Spain,** ***O*** **other,** ***MW*** **Molecular Weight**

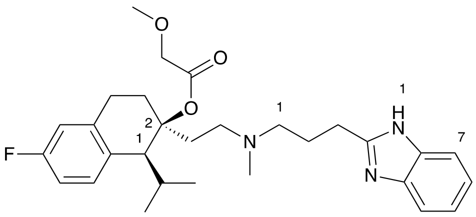
[a] **Not currently Food and Drug Administration (FDA) approved**

Observing the data in the previous tables (*Table 3* and *Table 4*) provided by a review from the *European Journal of Physiology*[25], there is proof that dihydropyridines used traditionally as antihypertensive agents can block TTCC with results of IC_{50} similar to traditional antiepileptic drugs. For instance, amlodipine (IC_{50} 0.031), aranidipine (IC_{50} 0.03-0.04) and azelnidipine (IC_{50} 0.04-0.07) show a nearby IC_{50} value of blockade as ethosuximide (0.3-1.0).

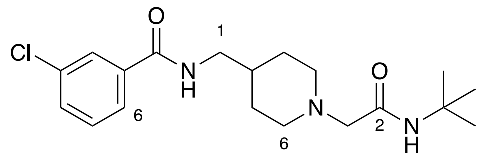
8.3.2. Novel T-type calcium channel antagonists

Inhibition of T-type calcium channels could be useful for the treatment of several central or peripheral diseases like pain, oncology, sleep disorders, Parkinson's disease or epilepsy[24] as well as playing an important role in neuroprotection[25]. Since pharmacoresistance to AEDs even in combination treatment is currently increasing[20], this calls for new compounds with new mechanisms of action. Especially, low-voltage-gated T-type calcium channels which are being assessed in several clinical trials[20]. First molecules discovered that claimed to be new TTCC blockers were Mibefradil (**1**) (currently withdrawn from the market)[34,20,24], Z-941 (**2**) and Z-944 (**3**), discovered by *Epirus Biopharmaceuticals*, are a piperidine-like molecule that showed efficacy in rodent models of seizure)[24,34,35], MK-8998 (**4**), finally assigned for the treatment of acute psychosis in patients with schizophrenia)[24,34,35], ABT-639 (**5**, finally suitable for the treatment of diabetic neuropathies)[24], TTA-P1 (**6**) and TTA-P2 (**7**), discovered by *Merck Pharmaceuticals*, has a piperidine-like molecular structure with good brain penetrance and suppression of seizures in the WAG/Rij rat model)[22] and TTA-A2 (**8**), identified by *Merck*, is the amide analogue of TTA-P compounds)[22].

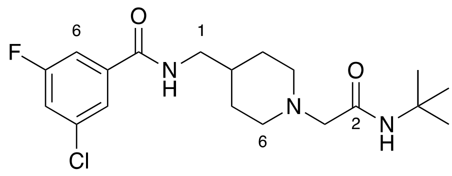
Figure 7. New T-type calcium channel blockers



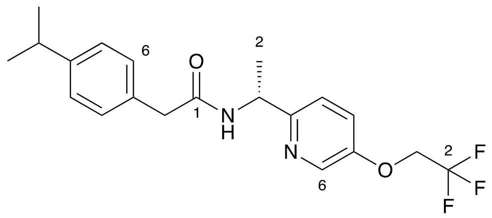
Mibefradil **1**



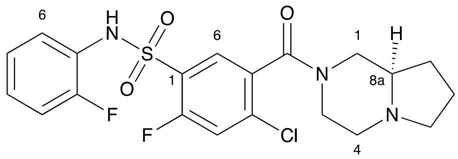
Z-941 **2**



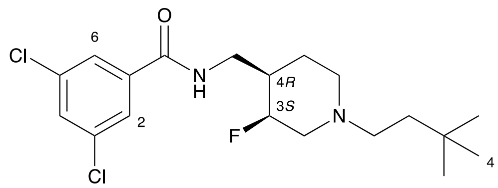
Z-944 **3**



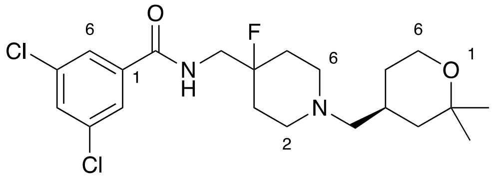
MK-8998 **4**



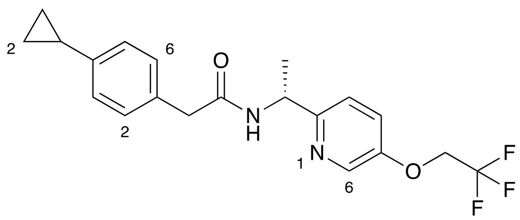
ABT-639 **5**



TTA-P1 **6**



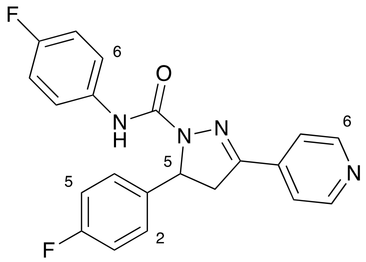
TTA-P2 **7**



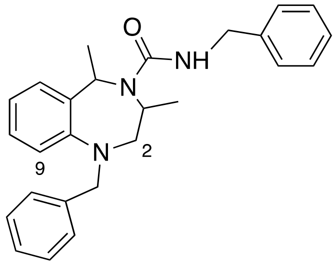
TTA-A2 **8**

On the one side, *Actelion Pharmaceuticals* identified a compound with a dihydropyrazole structure starting with the development of high-throughput FLIPR assays using similar conditions to other assays against three subtypes of Ca_v3 channels published previously. Optimisation of this molecule led to a series of dihydropyrazole compounds as potent, selective and brain penetrant TTCC blockers as well as having a good profile on cardiovascular safety (e.g. less hypotension as a side effect)[35]. Concretely, dihydropyrazole **9**, was used to evaluate potential efficacy and minimal safety issues[35]. However, issues related to solubility and intellectual property led to stop the development of this class of compounds[35]. Another attempt was the identification of new 1,4-benzodiazepines as brain penetrant and selective triple TTCC blockers. Starting from high-throughput screening (HTS) hit **10** with poor physicochemical and *in vitro* drug metabolism and pharmacokinetics (DMPK) properties of the molecule[24], they established a clear structure-activity relationship (SAR) to identify moieties allowing the improvement of its chemical properties. Optimisation led to the preparation of pyridodiazepine, resulting in a final **(3*R*, 5*S*)-11** structure by chiral HPLC separation of the racemic mixture[24].

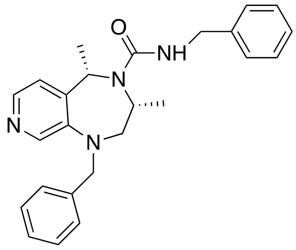
On the other side, *Idorsia Pharmaceuticals* discovered compound **12** (ACT-709478)[34], which has been selected as a clinical candidate, starting from the optimisation of derivatives from molecule *N*- (1-benzyl-1*H*-pyrazole-3-yl)-2-phenylacetamide[34].



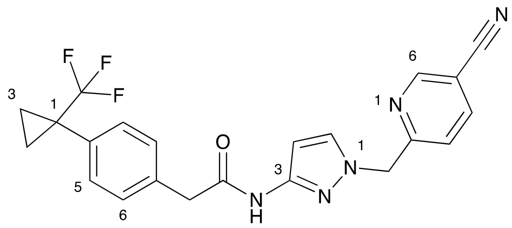
9



***rac*-10**



(3*R*,5*S*)-11



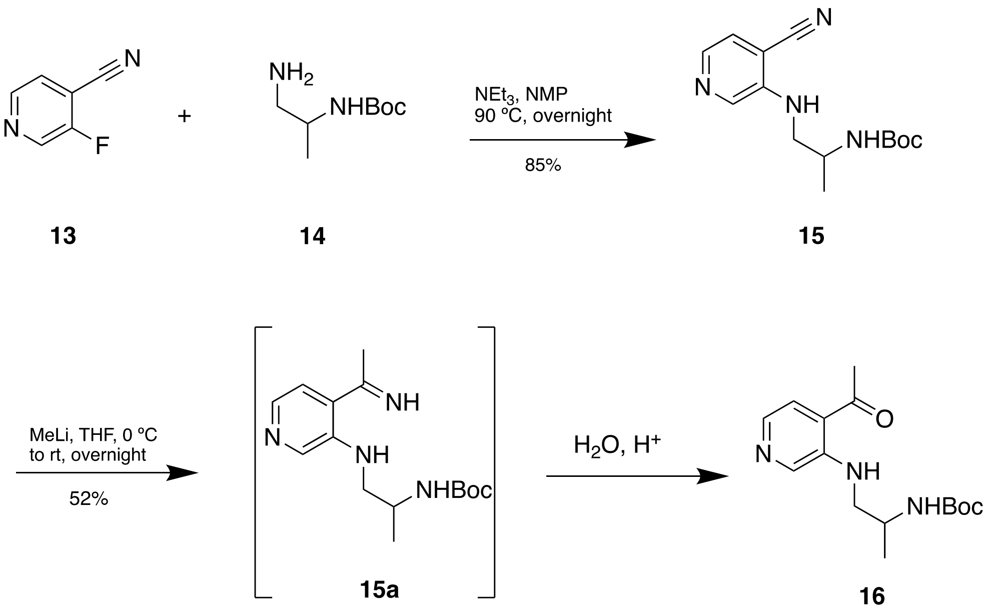
ACT-709478 **12**

8.3.2.1. Synthesis of compound (3R,5S)-8: a promising, efficient triple T-type calcium channel blocker

Actelion Pharmaceuticals have recently used a FLIPR assay within a HTS campaign[24] that led to the identification of a compound which was crucial for the discovery of **(3*R*,5*S*)-11** hit. This compound **10** showed a poor solubility profile, high lipophilicity and low metabolic stability[24]. The aim of their study was the maximum optimisation of this compound improving its pharmacokinetic profile based on changing different moieties of the original molecule. Different pathways of synthesis and its final products were compared in terms of biological activity on $Ca_v3.1$, $Ca_v3.2$, $Ca_v3.3$, solubility and brain penetration, which is measured by lipophilicity[24]. Final molecule **(3*R*,5*S*)-11** resulted highly effective in the WAG/Rij-rat model of generalised non-convulsive absence-like epilepsy[24].

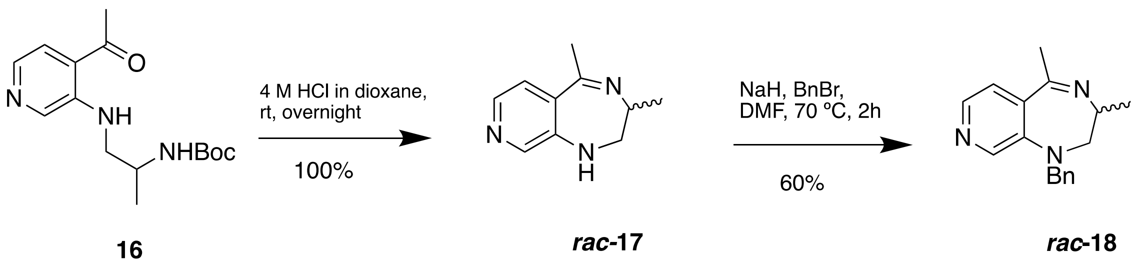
The first molecule **10** used as a hit to improve its properties was synthesised by the classical amide coupling-cyclization-reduction strategy[24]. Unfortunately, a non-benzodiazepine pyridine core was less potent and promising than a pyridodiazepinic core-like molecule[24]. Thus, another approach to reach the final molecule was an aromatic nucleophilic substitution (S_NAr) which was a 6 step sequence[24]. The synthesis of derivative **11** reported as an example, started reacting both commercial 3-fluoroisonicotinonitrile (**13**), primary amine **14** and triethylamine in NMP stirred at 90 ºC overnight, leading to pyridine **15**[24]. Water was added to the reaction mixture at room temperature and the precipitate was filtered and washed with water[24]. The solid was dissolved in DCM and washed again with water[24]. The organic layer was separated, dried in $MgSO_4$, filtered and further purified to give a white-like solid[24]. The solvents are usually removed under reduced pressure so that the pureness of the compound is optimised. Compounds number **13** and **14** can also be prepared with different reagents although being commercially available. Compound **15** was treated with MeLi in THF at a temperature of 0 ºC and, posteriorly, settled to room temperature overnight[24]. This reaction, which had medium output, led to a methylketone **16** (*Scheme 1*)[24].

Scheme 1. Synthesis of the Benzodiazepine Pyridine Core (S_NAR strategy)



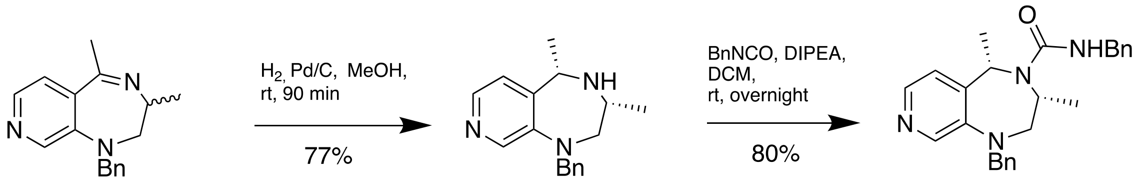
To the solution of (±)-*tert*-butyl-(1-((4-acetylpyridin-3-yl)amino)-propan-2-yl)carbamate (**16**) was added 4M HCl in dioxane and the solution was stirred at rt overnight until the Boc deprotection was completed, achieving compound ***rac*-17**[36]. The imine molecule was subsequently benzylated on the anilic nitrogen using benzylbromide in DMF and NaH as a base during 2h at 70 ºC, the reaction had a 60% output and turned out to molecule **18** (*Scheme 2*)[24]. Firstly, NaH was stirred at 0 ºC in DMF to a stirred solution of **17**, and secondly, after a 20 min stirring at 0 ºC, benzylbromide was added at 70 ºC for 2 hours[36]. The aqueous layer was adjusted to pH 10-11 and extracted with EtOAc. The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified as a yellow oil to give (±)-1-benzyl-3,5-dimethyl-2,3-dihydro-1*H*-pyrido[3,4-*e*][1,4]diazepine (**18**)[36].

Scheme 2. Boc deprotection and benzylation



In order to reach compound **19**, imine **18** was hydrogenated with the presence of the catalyst Pd/C, mixed in a solution with MeOH, stirred at room temperature during 1.5h and finally yielded **19**, a *cis/trans* compound with diastereoselectivity of 9:1[24]. The reaction had a 77% of output and the residue, once purified, gave ***cis*-19** after a column chromatography was used to separate both diastereoisomers. Compound ***cis*-19** was submitted to urea formation by adding benzyl isocyanate (BnNCO) and DIPEA at standard conditions overnight to achieve molecule **20**, which showed a high output of 80% (*Scheme 3*)[24]. The output of the reaction was 80%, and the residue was purified to yield **(3*R*,5*S*)-11**[36].

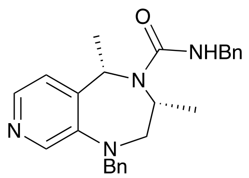
Scheme 3. Imine hydrogenation and urea formation

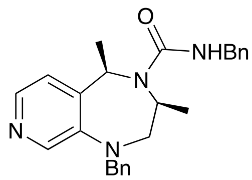


***rac*-18** ***rac-cis*-19** ***rac*-20**

To obtain the optimum enantiomer of molecule **20**, a chiral HPLC took place in order to separate **(3*R*,5*S*)-11** from **(3*S*,5*R*)-11**, whose appearance is presented as white solids. The effect of the inhibition on TTCC of both enantiomers is shown on the table below in terms of half maximal inhibitory concentration (IC_{50})[24].

Table 5. Activities of the enantiomers of molecule 11[24]





(3*R*, 5*S*)-11	**(3*S*, 5*R*)-11**
$Ca_v3.1$ IC_{50} = 138 ± 5 nM (n=3)	$Ca_v3.1$ IC_{50} = > 10000 nM (n=3)
$Ca_v3.2$ IC_{50} = 132 ± 36 nM (n=3)	$Ca_v3.2$ IC_{50} = > 11000 nM (n=3)
$Ca_v3.3$ IC_{50} = 11 ± 1 nM (n=3)	$Ca_v3.3$ IC_{50} = > 10000 nM (n=3)
HLM (μL/min/mg) = 56 (n=1)	
RLM (μL/min/mg) = 1156 (n=1)	

From compound number **20** until the discovery of the appropriated enantiomer as a clinical candidate, physicochemical properties were improved and *in vitro* DMPK were assessed in the WAG/Rij-rat model (generalised non-convulsive, absence-like epilepsy). Compound **20** was selected as a lead candidate and it displayed good solubility, improved HLM stability, moderate penetration of blood-brain barrier (BBB), unstable in terms of RLM and high exposure in plasma (C_{max}) after a pharmacokinetic *in vivo* study performed in WAG/Rij rat-model at a high dose of 100 mg/kg po. Moreover, a brain free fraction was calculated into brain concentration, and it corresponded approximately 0.25 times, 0.15 times, and 1.5 times the IC_{50} on the human Ca_v 3.1, Ca_v 3.2, and Ca_v 3.3 channels. Brain exposure may not be its strongest point, especially in Ca_v 3.1 and Ca_v 3.2 subtypes; however, molecule **20** was selected for proving its in vivo efficacy in the WAG/Rij-rat model of generalised non-convulsive absence-like epilepsy[24].

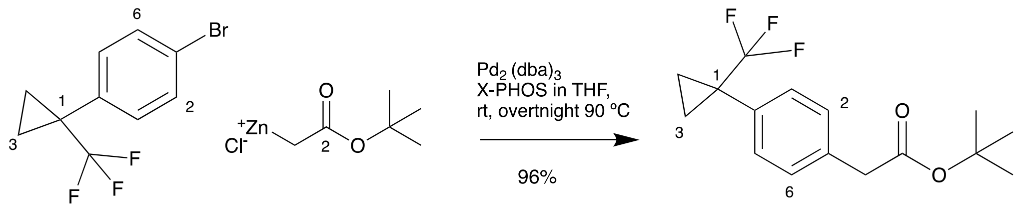
Molecule **11** was selected for a preparative chiral HPLC to increase the probability of *in vivo* efficacy[24]. Derivative **(3*R*, 5*S*)-11** was two times more active and stable in human liver microsomes (HLM) in comparison to ***rac*-11**. At the single oral dose mentioned before, these rats showed a decrease of 50% regarding the number of seizures and duration of them[24]. This study performed by *Actelion Pharmaceuticals* used telemetric transmitters which allowed a recording of the EEG[24]. To sum up, after SAR studies, *in vivo* pharmacological studies and DMPK properties, starting from the hit number **10** an improved molecule was discovered **(3*R*,5*S*)-11** showing a strong efficacy in the $Ca_v3.3$ T-type calcium channel (10 times stronger than $Ca_v3.1$ and $Ca_v3.2$)[24]. This results underline the importance and implication of this channel subtype in the pathophysiology of generalised epilepsies and suggest that the different channel variants in epilepsy would need a selective compound for one or the other T-type calcium channel subtypes[24].

8.3.2.2. Synthesis of compound ACT-709478: a new clinical candidate

Idorsia Pharmaceuticals reported the discovery of a new class selective, potent, brain penetrant T-type calcium channel blocker with a pyrazole carboxamide structure. One of the requirements for this molecule was an Ames negative result in the Ames test, which is biological assay used to identify revert mutations present in strains, substances[34,37], etc. by using bacteria to test whether a given chemical can give variations in the DNA of the test organism. Firstly, compound **28** *(see Scheme 6)* was identified as an Ames-negative aminopyrazole and was selected as a candidate to react with other successful compounds[34] and achieve an optimum molecule. After final optimisation, compound **24** *(see Scheme 5)* was identified and finally entered phase I clinical trials.

As shown in *Scheme 4*, *tert*-Butyl 2-{4-[1,1,1-(trifluoromethyl)cyclopropyl]phenyl}-acetate (**23**), is obtained by a mixture of 1-bromo-4-[1,1,1-(trifluoromethyl)-cyclopropyl]benzene (**21**), 2-*tert*-butoxy-2-oxoethyl-zinc chloride (**22**), $Pd_3(dba)_3$ and X-PHOS in THF at room temperature and stirred overnight at 90 ºC[34]. The reaction had an output of 96%. Moreover, another pathway to obtain compound **23** is using the commercially available product.

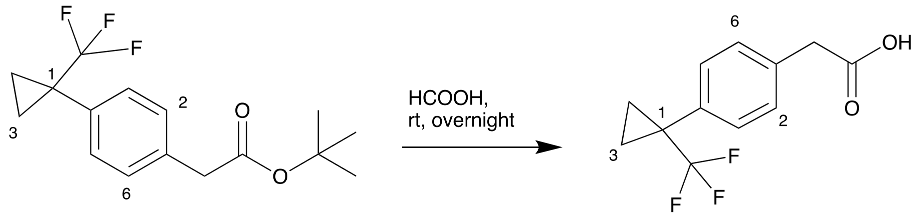
Scheme 4. Synthesis of compound 23



21 **22** **23**

To obtain carboxylic acid **24**, one of the main reagents, a solution of compound **23** is mixed with HCOOH at room temperature overnight (*Scheme 5*)[34]. As usual in these experimental sections, solvents are removed under reduced pressure.

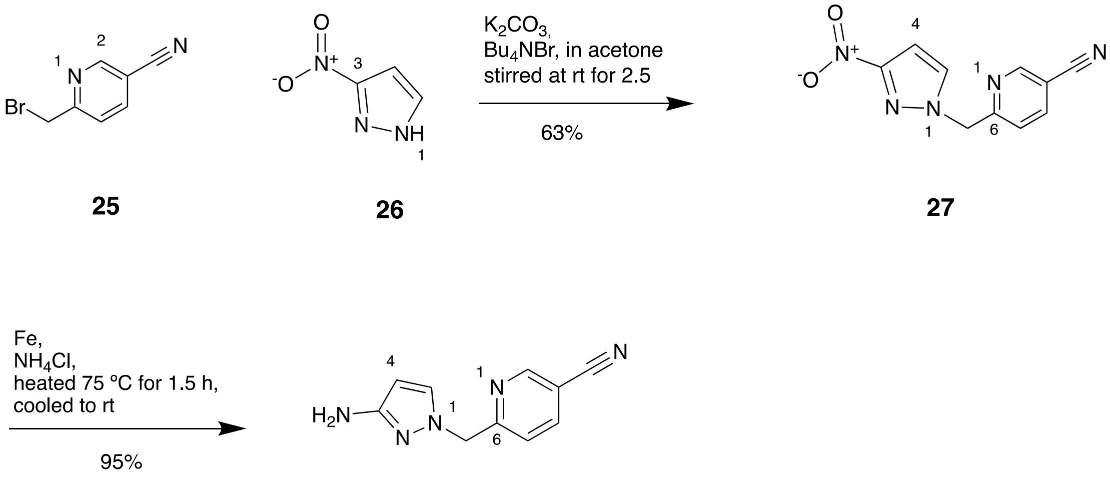
Scheme 5. Synthesis of compound 24



23 24

In order to synthesise molecule **28**, K_2CO_3, and Bu_4NBr in acetone were added into a mixture of 6-bromomethylnicotinonitrile (**25**) and 5-nitro-1*H*-pyrazole (**26**) at rt during 2.5 h. The reaction showed an output of 63% (*Scheme 6*)[34]. The resulting product **27** was filtered and rinsed with acetone and further purified by automated flash chromatography[34]. To a mixture of the resulting product, reagents Fe and NH_4Cl were added, and the mixture was heated at 75 ºC during 1.5 hours[34]. After cooling down at room temperature and yielding a 95%, molecule **28** was formed (*Scheme 6*)[34].

Scheme 6. Synthesis of molecule 28

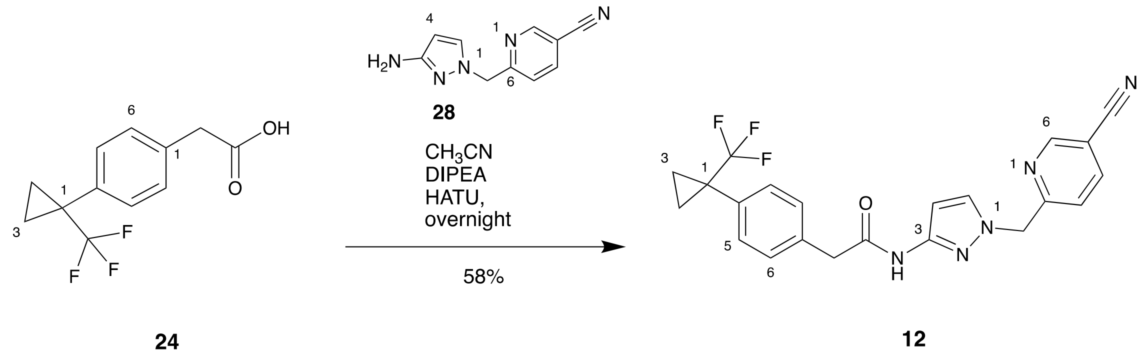


25 26 27

28

Finally, compound **24** and **28** were added into a mixture of CH_3CN, DIPEA and HATU, yielding a 58% (*Scheme 7*)[34] and leading to a final compound named ACT-709478 (**12**) The solution was stirred overnight, and the solvents were removed under reduced pressure[34].

Scheme 7. Formation of final compound 12 (ACT-709478)



The selectivity of this aminopyrazole (**12**) was evaluated against a wide human cardiac and neuronal ion channels where calcium channels are implicated, showing an inhibitory effect of compound **12** on currents through hCa_v3 type and a negative Ames test[34]. This compound was tested in mice, dog and cynomolgus TTCCs and showed a good blockade of $Ca_v3.3$ channels but with marked voltage-dependency[34]. It also blocked other calcium channels such as 1.2, 1.3 and 2.1 but with less potency[34]. The potential of compound **12** to cause drug interactions were evaluated by studying the inhibition of P-450 cytochrome and the inhibition potential of **12** was predicted to be low[34]. Referring to DMPK studies, PK of molecule **12** was assessed by predicting the clearance from microsomes (rat and human liver microsomes)[34]. Thus, the approach for a prediction of the human clearance in man was estimated, and both extrapolated clearance from HLM as well as RLM resulted in a low predicted clearance of compound ACT-709478[34]. Consequently, these results made the compound a new suitable candidate antiepileptic drug as a TTCC blocker.

9. CONCLUSIONS

Despite the numerous antiepileptic drugs currently available, it remains a wide resistance to the therapy of epilepsy. Increasing evidence from various studies indicates that T-type Ca^{+2} channels play a crucial role in neuronal excitability that underlies seizures. Bearing in mind that most of AEDs are seizure suppressors instead of being both seizure inhibitors and anti-epileptogenic, it urges to develop new compounds with high specificity on the different types of calcium channels ($Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$).

Firstly, two main compounds have been synthesised along the project whose therapeutic effect is improved towards the actual AEDs. Both are calcium antagonists. Since the three types of Ca^{+2} channels are widely expressed in the brain, these newer compounds act in a specific way, not only blocking the three of them but also showing a certain tendency to block one concretely. These molecules have reached Phase I clinical trials due to its potency, specificity and safety regarding to epilepsy treatment.

Secondly, whether the epilepsy is genetic or acquired, these new molecules have demonstrated in *in vivo* studies in animal models an excellent efficacy on the TTCC blockade. According to the predicted effect on human, researchers from *Actelion Pharmaceuticals* and *Idorsia Pharmaceuticals*, extrapolated data from the animal models and thus, results obtained were promising for becoming new candidates in epilepsy treatment. Molecule **(3*S*,5*R*)-11** discovered by *Actelion Pharmaceuticals* was obtained from optimisation of a hit with poor physicochemical and DMPK properties. In recent studies, the racemic mixture of molecule **11** showed excellent HLM stability and thus, was elected for *in vivo* efficacy studies. The separation of the racemic mixture by chiral HPLC demonstrated the difference between both enantiomer properties, being **(3*S*,5*R*)-11** the most suitable compound for further investigation. Regarding to specificity, this pyridodiazepine showed modest activity on $Ca_v3.1$, $Ca_v3.2$ while $Ca_v3.3$ was strongly blocked on the WAG/Rij rat model, highlighting the importance and implication of $Ca_v3.3$ in the pathophysiology of generalised epilepsies.

Thirdly, molecule **12** was obtained by *Idorsia Pharmaceuticals* and displayed excellent properties allowing this aminopyrazole to enter phase I clinical trials. By making react two final molecules **24** and **28** in specific conditions and reagents, compound ACT-709478 was finally obtained. In the same manner as molecule **(3*S*,5*R*)-11**, metabolic stability in liver microsomes was studied as well as the unbound fraction in plasma, pharmacokinetics, *in vivo* brain penetration potential, drug interaction and other safety studies. Different classes of TTCC were studied and, in comparison to *Actelion Pharmaceuticals* whose purpose was to identify a substance capable of blocking one specific Ca^{+2} channel, *Idorsia Pharmaceuticals* wanted to develop a molecule blocking all three TTCCs with minimal divergences between their potencies.

Finally, the implication of TTCCs in many diseases led to a need to identify truly selective TTCC antagonists. In epilepsy, the overexpression of these channels contributes to a high predisposition to epileptogenesis. This fact suggested a new drug discovery programme on novel, potent and more selective TTCCBs used in cardiovascular diseases and now approached to the epilepsy field. These two molecules became promising clinical candidates for a new strategy on generalised epilepsy treatment.

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